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The Use of Transposons to Introduce Well-Defined Deletions in Plasmids: Possibilities for *in Vivo* Cloning

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A method for obtaining well-defined deletions in an octopine Ti plasmid was developed. It was based on the assumption that deletions would occur between two directly repeated transposons, when both are temporarily present in one plasmid molecule. To obtain such a situation, recombination has been forced between *Agrobacterium tumefaciens* Ti plasmids, each carrying the transposon Tn1831 at a different position. In a number of cases, most probably when the transposons are directly repeated, deletion formation indeed occurred and at high frequency. Mutants were isolated carrying Ti plasmids with one copy of Tn1831, and the region of DNA, between the positions of the transposons in the original plasmid, deleted. Moreover, in the case that the segment of DNA, enclosed by the two transposons, harbors the requirements for autonomous replication of an R plasmid, it is shown that *in vivo* cloning of such a segment of the Ti plasmid on the R plasmid can be accomplished.

It has been well established that the Ti plasmid of *Agrobacterium tumefaciens* is essential for crown gall tumor formation on dicotyledonous plants (*13,14*). Part of this plasmid, which is called the T-DNA region, is stably integrated and at least partly transcribed in the plant tumor cell (*1,3,5,10*). A physical and a gross genetic map of an octopine Ti plasmid (M_r 121 Md) has been constructed (*2,9*).

Using transposon Tn1831 (M_r 11 Md), which carries determinants for resistance against spectinomycin (Sp),[1] streptomycin (Sm), and mercury chloride (Hg), several insertions into the octopine Ti plasmid have been isolated and their sites of insertion have been determined (*6,12*). Some of the Ti::Tn1831 plasmids appear to be affected in the capacity to confer virulence to their host (*6*), as was also reported for Ti::Tn904 plasmids (*8,11*) and Ti::Tn5 plasmids (*4*). Genetic complementation studies with these mutant Ti plasmids would be useful for the analysis of functions involved in tumor induction. A method to direct the occurrence of well-defined deletions in Ti plasmids would aid such studies, especially when this method could also be used for the construction of R primes, which are compatible with Ti plasmids. To find such a method was the aim of the present work.

The method we have developed, and which is schematically presented in Fig. 1, is based on the following reasoning: (i) When a Ti plasmid, carrying a Tn1831 insertion, is introduced into a bacterium with a Ti plasmid also having a Tn1831 insertion, but at another position, recombination can occur. In the case of a double crossover this will give rise to an intermediate (hypothetical) Ti plasmid, harboring two identical transposons (Fig. 1c). Since the plasmids are incompatible, only one plasmid will maintain in the absence of selection. (ii) When both transposons are directly repeated, homologous recombination between them will generate two circular DNA molecules, each with one copy of Tn1831. The one lacking a functional replicator is lost. The

[1] Abbreviations used: Sp, spectinomycin; Sm, streptomycin.

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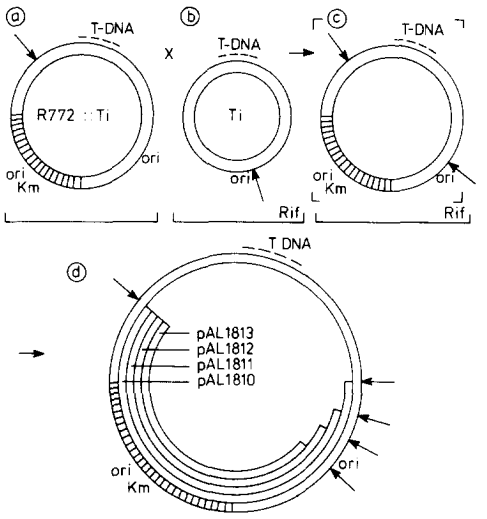

FIG. 1. Scheme for the induction of well-defined deletions. A strain carrying the cointegrate plasmid R772::(Ti::Tn1831) (a) was crossed on rich medium for 24 h with a strain carrying a Ti::Tn1831 plasmid (b). These two plasmids are incompatible (*7*). Homologous recombination between the two plasmids can occur. This is assumed to result in a cointegrate plasmid carrying a second copy of Tn1831 (c). Recombination between the two directly repeated transposons will delete the region located between these transposons. In all cases, the Ti-plasmid DNA fragments, which were expected to be deleted, carried genes that code for octopine catabolism. Approximately 1% of 10^6 transconjugants turned out to be unable to catabolize octopine and, when tested, were avirulent on tomato. Plasmid DNA was isolated from these strains, and characterized using restriction enzymes and subsequent agarose gel electrophoresis of the fragmented DNA (data not shown). In (d) we show a number of deleted Ti plasmids, including an R prime, obtained by following this procedure. Arrows indicate the position of a Tn1831 insertion. The striped part of the circle represents the R772 part of a cointegrate plasmid. Abbreviations: ori, origin of replication; Km, resistance against kanamycin; Rif, resistance against rifampicin.

other one is a Ti plasmid, deleted in the region between both copies of Tn1831 in the hypothetical intermediate (Fig. 1d). If this region contains genes for octopine catabolism, *occ*-genes, then these Ti-plasmid deletion mutants can be selected for by their Occ$^-$ phenotype. When the transposons are invertedly repeated, no deletion formation will occur. This means that, in doing these experiments, it can be expected that certain Tn1831 combinations will not lead to a deletion in between their positions. In addition, if one of the Ti plasmids does not contain a Tn1831, no well-defined deletions are expected to occur. (iii) When one of the Ti plasmids, which are brought together, consists of a cointegrate with an R plasmid (Fig. 1a), then the hypothetical intermediate can fall apart in two functional plasmids. This occurs when the R plasmid is located in the segment which does not have the Ti replicator.

In the experiments a donor strain carrying a cointegrate plasmid, consisting of the broad host range IncP-1-type plasmid R772 (coding for Km^r), and an IncRh-1 octopine Ti-plasmid (Ti::Tn1831) is used. The donor strain was crossed on rich medium with recipient strains, each carrying a Ti::Tn1831 plasmid, in which Tn1831 was inserted at a different position. In all cases the segment of DNA, expected to be deleted, carried genes that code for octopine catabolism. After crossing we selected for the chromosomally encoded resistance of the recipient (Rif^r) and for a cointegrate plasmid marker (Km^r) of the donor. The strains used in the conjugation experiments as well as the percentage of transconjugants with an Occ$^-$ phenotype, isolated in the different crosses, are presented in Table 1. The different Tn1831 positions in the Ti plasmid of these strains is drawn in Fig. 2. Approximately 1% of the transconjugants from such a cross showed an Occ$^-$ phenotype and, these were found to have the predicted deletion. In several other crosses, however, no deletion formation was found to occur, as was expected for a situation in which the two transposons are invertedly repeated. If the plasmid of the donor, or the plasmid of the recipient, did not contain a Tn1831 insertion, no deletion formation was observed either. This all indicates that deletion formation is dependent upon the presence and probably also on the orientation of Tn1831. However the actual mechanism by which the well-defined deletion formation takes place, i.e., whether the hypo-

TABLE 1

CROSSES AND TRANSCONJUGANTS OBTAINED

Donor[a]		Recipient[b]		Percentage of transconjugants with Occ⁻ phenotype	Collection number of mutant Ti plasmid
Strain	Ti plasmid	Strain	Ti plasmid		
LBA973	pAL969[c]	LBA1698	pAL1698	0	—
LBA1821	pAL1802	LBA677	pTi	0	—
LBA1821	pAL1802	LBA1664	pAL1664	0	—
LBA1821	pAL1802	LBA1673	pAL1673	0	—
LBA1821	pAL1802	LBA1634	pAL1634	1	pAL1810
LBA1821	pAL1802	LBA1698	pAL1698	1	pAL1811
LBA1821	pAL1802	LBA1625	pAL1625	1	pAL1812
LBA1821	pAL1802	LBA1666	pAL1666	1[d]	pAL1813

[a] All donors are gentamycin resistant.

[b] All recipients are rifampycin resistant.

[c] Plasmid pAL969 is the same as plasmid pAL1802, except that it does not contain the transposon Tn1831.

[d] Occ⁻ phenotype was detected after crossing the transconjugants with LBA2228 (Gen^r, containing no Ti plasmid).

thetical intermediate also occurs, is not yet known. For the moment, the intermediate at least easily visualizes the predictable region where deletions will occur, and is consistent with the results obtained.

Especially for large plasmids our approach may be useful for the localization and characterization of plasmid-encoded functions. What one needs is, first, a plasmid which is Tra$^+$, second, a number of plasmids with one and the same transposon inserted at different places, and third, an extra selectable marker on the donor plasmid.

We have also applied our approach for *in vivo* cloning of a Ti plasmid fragment on an R plasmid. To this end, the R772 part of the

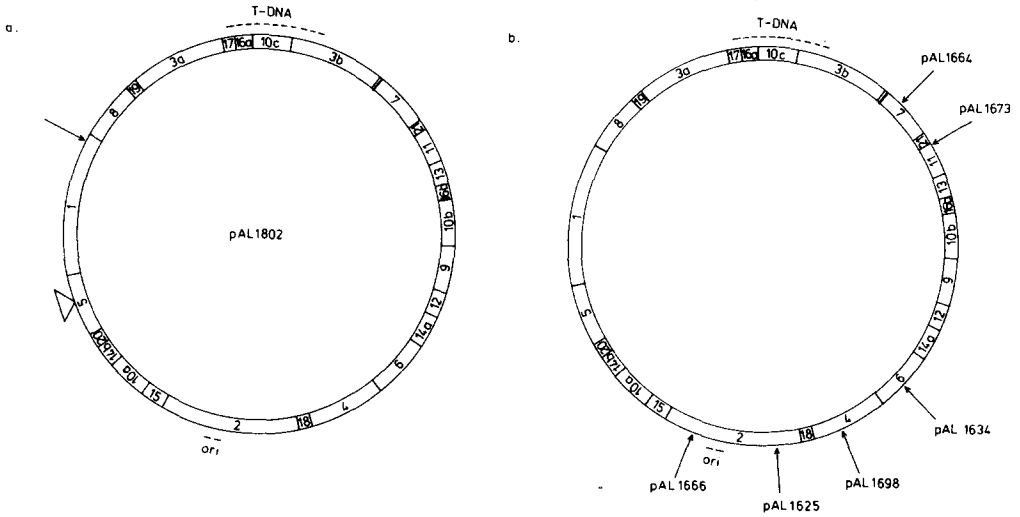

FIG. 2. Organization of Ti plasmids used in the experiments. The physical map for the restriction enzyme *Sma*I is presented. (a) The plasmid of the donor strain. (b) The plasmids of the recipient strains. An arrow indicates the position of a Tn1831 insertion. The triangle on the plasmid of the donor represents the integration place of R772 into the Ti plasmid.

cointegrate plasmid has been enclosed between two copies of Tn1831 in the hypothetical intermediate, i.e., LBA1821 was crossed with LBA1666 (see Table 1 and Fig. 2). In this case the transposon-induced deleted segment, not carrying the Ti-ori, is not lost, but is stably maintained and replicated in *Agrobacterium tumefaciens*, because it can use the R772-replicator. This R772-derived plasmid (pAL1813, see Fig. 1) carrying a defined region of the Ti plasmid, behaves as the wide host range R772 plasmid; e.g., it is compatible with Ti plasmids and is stably maintained in *E. coli*.

Using *in vitro* techniques, cloning of DNA fragments bigger than 30 Md is rather difficult, but sometimes desirable. For large fragments the described procedure may be more suitable than *in vitro* cloning.

Currently we are isolating more *in vivo* constructed R plasmids, carrying different segments of the octopine Ti plasmid, which are compatible with Ti plasmids. Together with pAL1813, these plasmids will be used in complementation studies of Ti plasmids affected in the capacity to confer virulence on their host. The first results suggest that complementation of Ti-plasmid-encoded virulence functions is possible.

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